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# Chromatographic fingerprinting and free-radical scavenging activity of ethanol extracts of *Muntingia calabura* L. leaves and stems

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ABSTRACT

**Objective:** To determine the thin-layer chromatography (TLC) fingerprint profiles and to evaluate the *in vitro* antioxidant activity of ethanol extracts of *Muntingia calabura* (*M. calabura*) leaves and stems.

**Methods:** The leaves and stems were extracted using ethanol as solvent. The TLC separation of the phytochemical constituents of the leaf and ethanol extracts was carried out in ethyl acetate: *n*-hexane and chloroform: ethyl acetate mobile phase systems. Distinct spots were visualized under visible light, UV 254 nm, UV 366 nm and after spraying with vanillin-sulfuric acid. The 2,2-diphenyl-1-picrylhydrazyl free-radical scavenging assay was used to evaluate the antioxidant activity of the extracts.

**Results:** Both the leaf and stem ethanol extracts at 4 mg/mL exhibited 2,2-diphenyl-1-picrylhydrazyl inhibition of more than 90%, relative to gallic acid. The results of TLC showed that the degree of resolution between the constituent spots was comparable between the two mobile phase systems using the different visualization wavelengths. Under the 254 nm visualization, few spots were observed in leaf and stem extracts. Visualization at 366 nm yielded the greatest number of observable spots of various colors in both leaf and stem extracts. More spots were visualized upon post-derivatization with vanillin-sulfuric acid in the TLC chromatograms using chloroform: ethyl acetate mobile phase, compared to those in ethyl acetate: *n*-hexane mobile phase.

**Conclusions:** *M. calabura* exhibited very high antioxidant activity in its leaves and stems ethanol extracts, both of which are used in traditional medicine. The TLC results demonstrated the presence of diverse secondary metabolites in the leaf and stem ethanol extracts, indicating that the antioxidant activity, including other bioactivities may be attributed to these phytochemical constituents. This paper has reported for the first time the TLC fingerprinting of *M. calabura* using visible light, UV 254 nm, UV 366 and post-derivatization with vanillin-spray to visualize separate spots on TLC plates.

## 1. Introduction

*Muntingia calabura* L. (*M. calabura*) is a tropical tree species indigenous in the Central and Southern Americas. It is also widely distributed throughout Southeast Asia. In the Philippines, it is known locally as “aratiles”. Berries from this tree are edible and used as supplements in local diets, while leaves are consumed in a decoction similar to tea [1]. Moreover, there are documented traditional medicinal functions of the various organs of this plant, including fruits, flowers, leaves, and stems [2].

Previous studies have demonstrated the various modes of action of this plant. Aqueous extracts from the leaves have shown anti-nociceptive properties, modulated by the L-arginine/nitric oxide/cyclic guanosine monophosphate pathway [3–5]. Methanolic and ethyl acetate fractions of the leaf crude extracts were shown to inhibit the growth of both methicillin-

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sensitive *Staphylococcus aureus* and multidrug resistant *Staphylococcus aureus*. Four known flavonoids were found to be the active antimicrobial constituents [6,7]. Cytotoxic activities against various leukemia cell lines [8], colorectal and lung carcinoma cell lines [6,9] were also observed from crude and fractionated leaf extracts.

The secondary metabolite classes present in the crude extracts of *M. calabura*, predominantly the flavonoid class, are known to be responsible for the plant's various bioactivities [10]. Rapid methods for systematic characterization and fingerprinting need to be developed to facilitate research into these compounds. Fingerprinting methods will also allow the positive identification of the source material, any active principals, and possible adulterants [11]. This study presents a report of the visualization of the phytochemical constituents of *M. calabura* leaves and stems using thin-layer chromatography (TLC) under two mobile phase regimes; the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging activity of both organs is also presented.

## 2. Materials and methods

### 2.1. Plant material and crude extract preparation

Leaf and stem specimens were identified and authenticated at the Jose Vera Santos Memorial Herbarium at the Institute of Biology, University of the Philippines. Samples were air-dried for two weeks before pulverization. These were then separately pulverized and immersed in 95% ethanol (1:10, w/v) for 72 h, filtered, and concentrated via rotary evaporation (Laborota 4001, Heidolph). Concentrates were further air-dried for 7 days. Crude extracts were stored in sealed containers until further analysis.

### 2.2. TLC analysis

The stationary phase was plain 5 cm × 17 cm pre-coated silica gel plates (TLC grade, Merck, Darmstadt, Germany). Ethyl acetate: *n*-hexane (70:30, v/v), and chloroform: ethyl acetate (60:40, v/v) were used separately as mobile phase systems. The chromatograms were visualized in visible light, short-wave UV at 254 nm, and long-wave UV at 366 nm. Plates were then subjected to vanillin-sulfuric acid spray in visible light for post-derivatization [12]. The following formula was used to obtain the $R_f$ value for each visualized spot:

$$R_f = \frac{\text{Distance travelled by spot}}{\text{Distance travelled by solvent front}}$$

### 2.3. Free radical scavenging assay

The modified DPPH assay by Rameshkumar and Sivasudha was used to evaluate the antioxidant activity of the extracts [13]. A 300 μmol/L solution of DPPH in absolute ethanol was prepared. The solution at 95 μL was then dispensed to 96-well microtiter plates. Gallic acid was used as positive control while dimethylsulfoxide (DMSO) was used as negative control. Five microliters of the controls and extracts were each added to the wells to constitute a final volume of 100 μL. The plate was then incubated at 37 °C for 45 min and absorbance (Abs) at 570 nm was read post-incubation. Free radical inhibition activity of the extracts was calculated using the following formula:

$$\%\text{Inhibition} = \frac{\text{Abs}_{\text{DMSO}} - \text{Abs}_{\text{Extract}}}{\text{Abs}_{\text{DMSO}} - \text{Abs}_{\text{Gallic acid}}} \times 100$$

## 3. Results

### 3.1. TLC analysis

The thin-layer chromatograms are shown in Figures 1–4. Leaf ethanolic extracts uniformly yielded more spots, compared to stem ethanolic extracts in both solvent systems.

In general, the degree of resolution between the constituents was comparable between the two mobile phase systems. Under illumination at 254 nm, fewer spots for both extracts and mobile phases were observed. Visualization at 366 nm yielded the greatest number of observable spots, 15 (ethyl acetate: *n*-hexane) and 16 (chloroform: ethyl acetate) spots of various colors were observed on the leaf extracts, while 10 (chloroform: ethyl acetate) and 11 (ethyl acetate: *n*-hexane) were found on the stem extracts. Upon post-derivatization with vanillin-sulfuric acid spray, more spots were visualized with chloroform: ethyl acetate mobile phase. There are observable overlaps in the methods of visualization used.

### 3.2. Antioxidant activity

The DPPH free-radical scavenging assay was used to approximate the antioxidant activity of the leaf and stem

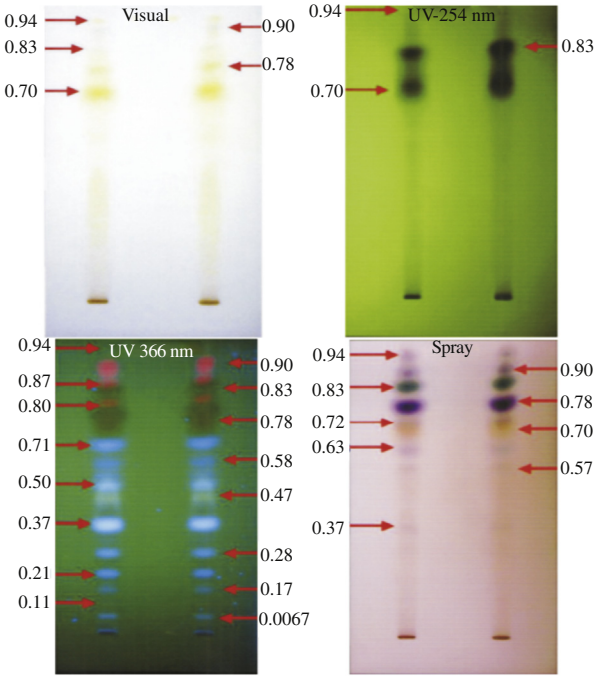


**Figure 1.** Chromatograms from leaf extracts of *M. calabura* in chloroform: ethyl acetate (60:40, v/v) mobile phase, visualized under visible light, 254 nm, 366 nm, and post-derivitization with vanillin-sulfuric acid spray. $R_f$ values are indicated.

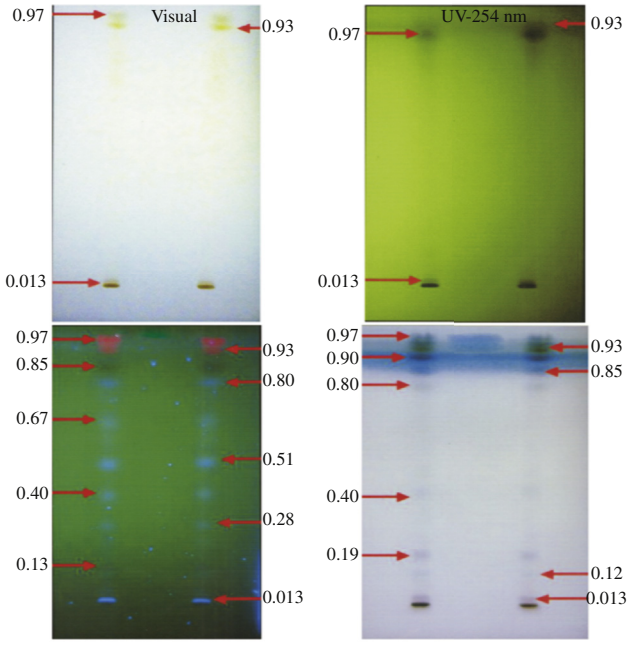


**Figure 2.** Chromatograms from stem extracts of *M. calabura* in chloroform: ethyl acetate (60:40, v/v) mobile phase, visualized under visible light, 254 nm, 366 nm, and post-derivitization with vanillin-sulfuric acid spray. $R_f$ values are indicated.

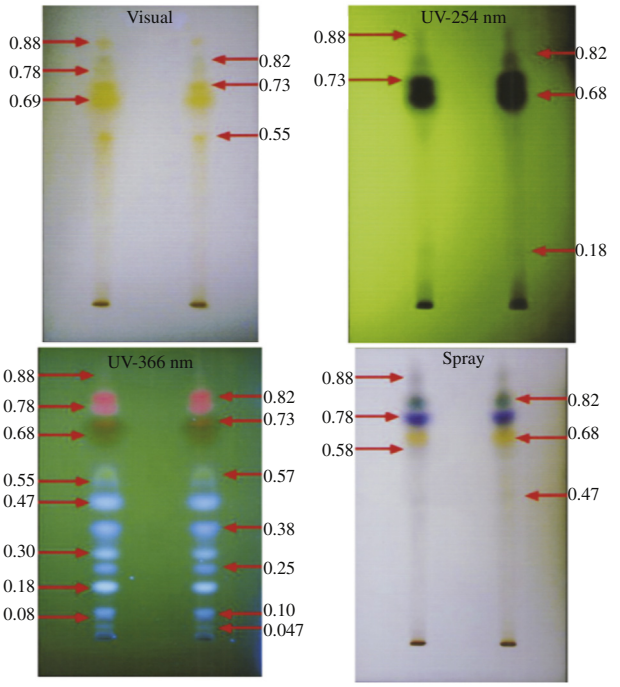


**Figure 3.** Chromatograms of *M. calabura* leaf ethanolic extracts in ethyl acetate: *n*-hexane (7:3, v/v), visualized under visible light, 254 nm and 366 nm UV light, and visible light after post-derivatization with vanillin-sulfuric acid spray. $R_f$ values are indicated.

ethanol extracts. Both extracts at 4 mg/mL demonstrated considerable DPPH inhibition of relatively high values above 90%. The leaf ethanol extract demonstrated (99.1 ± 2.5)% inhibition, relative to 4 mg/mL gallic acid positive control. The stem ethanol extracts were significantly lower at (93.9 ± 2.2)% inhibition.

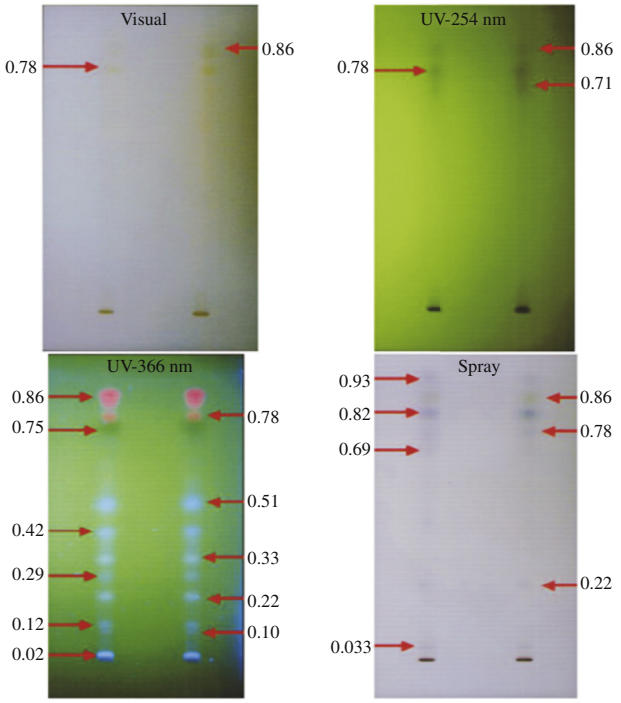


**Figure 4.** Chromatograms of *M. calabura* stem ethanolic extracts in ethyl acetate: *n*-hexane (7:3, v/v) visualized under visible light, 254 nm and 366 nm UV, and visible light after post-derivatization with vanillin-sulfuric acid spray. $R_f$ values are indicated.

## 4. Discussion

A free radical is a chemical species which contains one or more unpaired electrons in its outermost orbital. In biological contexts, reactive oxygen species (ROS) include free radicals derived from molecular oxygen; it also includes hydrogen peroxide ($H_2O_2$) owing to its role in the production of other ROS [14]. A similar collective term, reactive nitrogen species (RNS), is also used for products of nitric oxide and the superoxide anion ($O_2^{-\bullet}$). These are highly reactive and are linked to various diseases; there are, however, known endogenous functions of certain ROS [15,16]. Both ROS and RNS are known to alter DNA structure, disrupt cellular signaling pathways, and modulate proteins and genes for stress response. Together, these effects may contribute to carcinogenesis [17].

Reactive oxygen species (ROS) are produced *in vivo* via both enzymatic and non-enzymatic processes [18]. In the mitochondria, superoxides are produced on both the outer and inner mitochondrial membranes, and in the mitochondrial matrix. In the mammalian complex I, the superoxide anion ($O_2^{-\bullet}$) may be produced in response to a high NADH/$NAD^+$ ratio in the matrix. In addition, a high proton motive force ($\Delta p$) and a reduced coenzyme Q pool may induce reverse electron transfer, partially reducing $O_2$ to $O_2^{-\bullet}$ [14,19]. The superoxide anion may then form hydrogen peroxide ($H_2O_2$) through dismutation [19].

Several strategies are employed intracellularly to convert ROS and RNS into less deleterious compounds. Superoxide dismutases (SOD) are a family of enzymes which play a key antioxidant role. It converts $O_2^{-\bullet}$ to $H_2O_2$. Two enzymes, catalase and glutathione peroxidase work in conjunction with SOD to eliminate $H_2O_2$. MnSOD is found mainly in the mitochondrial matrix, while CuZnSOD is found in the intermembrane space and in the cytosol. Glutathione peroxidase catalyzes the

oxidation of reduced glutathione to glutathione disulfide using $H_2O_2$ [14,20]. Catalase, on the other hand, decomposes $H_2O_2$ to $H_2O$ and $O_2$; it is mainly found in peroxisomes. Cytochrome *c* is reduced by $O_2^{-\bullet}$, which generates $O_2$ [20].

Antioxidants are also found in dietary sources. Wiseman and Halliwell observed that there appears to be a decrease in various types of cancers along the gastrointestinal tract. There is evidence that ascorbate, commonly found in food, inhibits the formation of carcinogenic *N*-nitroso compounds [21]. Plant natural products are a rich source of antioxidant compounds, many of them belonging to the flavonoid and other phenolics groups of secondary metabolites. Some of these plant products have also been suggested to play a role in cancer prevention [22,23].

A convenient and fast method of measuring free radical scavenging activity, the DPPH assay employs the stable free radical DPPH•. In solution, DPPH• is a stable purple free radical which changes color to yellow upon reduction by antioxidants [24]. The presence of antioxidants in solution quenches the DPPH• free radicals through the donation of hydrogen atoms or electrons [25].

There is a need for the chemical profiling of the secondary metabolites from various naturally derived medicinal substances in aid of rapid identification and standardization. The detection of common adulterants and additives in supplements also benefits from chemical characterization [26]. This process is performed through a combination of chromatographic methods. TLC is an indispensable tool in the identification, characterization and subsequent standardization of natural products. For known pharmaceutical formulations, TLC profiles provide qualitative information on the presence and absence of particular metabolites [27]. In combination with densitometric methods, TLC may also provide rapid and satisfactory quantification. Moreover, a study comparing high performance liquid chromatography and high performance thin-layer chromatography densitometry demonstrated no statistical differences between accuracy, precision and repeatability between the two methods [28]. Another study on the identification and quantification of sibutramine, an illegal additive in some slimming drugs found comparable results using both methods [26].

In this study, the profiles obtained demonstrated the diversity of secondary metabolite constituents found in *M. calabura*. In general, more spots were visualized from the leaf ethanolic extracts under visible light, UV 366 nm, UV 254 nm, and the post-derivatization with vanillin-sulfuric acid spray. In addition, spots common to both leaf and stem extracts with similar visualization and $R_f$ values were observed. In particular, these spots were found at $R_f$ values ranging from 0.51 to 0.93, although highly polar compounds were also visualized at 0.02 to 0.30. It can be conjectured that these spots may be identical or belong to the same metabolite class. Substances containing aromatic moieties nominally absorb at 254 nm, and are easily detected when illuminated at this wavelength. Phenolic compounds, which include tannins and flavonoids, may be visualized at this wavelength. In addition, fluorescing substances such as chlorophylls may be visualized using longwave UV irradiation at 366 nm [29].

In conclusion, the leaves and stems *M. calabura* has been found to demonstrate very high antioxidant activity, both of which are used traditionally in treating various ailments. Moreover, TLC has demonstrated the presence of a highly diverse assemblage of secondary metabolites in the extracts. This suggests that antioxidant activity, including other bioactivity of the plant may be attributed to the presence of these secondary metabolites. *M. calabura* has a great potential to be a source of novel antioxidant and drug compounds. More work is currently being done to purify, isolate, and identify the active antioxidant components.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgments

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